To Director General, JSPS

ID No. US 04015

JSPS FELLOWSHIP FOR RESEARCH IN JAPAN

FINAL RESEARCH REPORT*

Name of Fellow: Edward F. Orlando, Ph.D.

Affiliation: St. Mary's College of Maryland

Name of Host Institution: National Institute of Basic Biology (NIBB), Okasaki

Name of Host Scientist: Professor Taisen Iguchi

Fellowship Period: July 2 – July 24, 2004

Title of Research in Japan: Gene cloning and expression in the mangrove rivulus, *Rivulus marmoratus*

Date: August 21, 2004 (original submission date)
May 31, 2005 (final submission date)*

***This is the final report, including data, from the research completed at Professor Taisen Iguchi's lab, NIBB, Okasaki, Japan. I apologize for submitting this final report so late, but two traumatic events took place in my personal life since completing the fellowship. My wife and I had twins in mid-October, and my mother became gravely ill almost concurrently. Together with my responsibilities at the College, helping to take care of our newborn children and visiting my mother and father each month took all of my effort. This delay in submitting the final report should in no way dilute my gratitude for being accepted to participate in the program. The fellowship has not only enabled this research study (which has culminated in a minimum of two manuscripts, with one currently in preparation) but has begun what I hope are life-long cooperative relationships between my lab in the USA and Dr. Iguchi's lab in Okasaki and Dr. Kanamori's lab in Nagoya, Japan.**

Fellow's signature: 

Office/Home: (240) 895-4376/ (410) 474-1097
Fax No.: (240) 895-4996
Email: eforlando@smcm.edu
Address: St. Mary's College of Maryland, 18952 E. Fisher Road, Biology Department, St. Mary's City, MD 20686 USA

Research Conducted at Professor Taisen Iguchi's Lab, NIBB

The picture to the right shows the Iguchi Lab at the National Institutes of Bioenvironmental Science, Okazaki. Although all members of the lab were helpful and welcoming to me during my fellowship, there were four people in particular who directly helped me and I would like to thank them formerly in this report. Colleagues at the Iguchi Lab who assisted me with my research during the JSPS fellowship include Yoshinao Katsu, Ph.D. (research associate, C) and Shinichi Miyagawa (Ph.D. earned Spring 2005, D), Taeko Imaizumi (secretary, B), and Professor Taisen Iguchi (A). Finally, I thank the Japanese Society for the Promotion of Science its generous support of my research fellowship.

Research Introduction

Among vertebrates, the mangrove rivulus (*Rivulus marmoratus*)it is the only known self-fertilizing hermaphrodite (Harrington, 1961). Rivulus is a marine teleost endemic to mangrove estuaries, and is distributed from Florida (USA) to Brazil and throughout the Caribbean Sea, and is one of only two fish species which spend their entire life cycle within the mangrove estuary (Taylor, 2000). Most rivulus collected from the wild are hermaphrodites (herms), yet in some populations males are found (only a few males have ever been collected in Florida, yet up to 25% of fish sampled are males in Carrie Bow Cay, Belize) (Turner, *et al.*, 1992). In the laboratory, sex determination is known to be affected by temperature as males can be induced to develop by raising embryos at $< 20^0$C, whereas the normal culture temperatures of 25^0C yields hermaphrodites (Harrington, 1975). Interestingly, no one has ever observed the differentiation of sexually mature females, thus, the functional relevance of males is unknown at this time. Since the NSF-JSPS fellowship, my students and I have induced the differentiation of females and males using estrogen and androgen treatments, respectively.

My overall objectives for the fellowship research were to (1) increase the scope of questions that I can address by gaining competence at using molecular techniques relevant to gene expression, e.g., quantitative RT-PCR (QPCR) and (2) to develop a professional relationship with Professor Iguchi that will serve both labs and encourage future international collaborations. My specific goals were to clone the ERs, AR, aromatases, and dmrt-1 genes in Rivulus and then compare the expression of these genes using QPCR in the brain, liver, and gonadal tissues of herms and primary male rivulus.

Research Results and Discussion

My fellowship was very successful. During my three weeks at the lab, Dr. Katsu and I partially cloned the brain and gonadal aromatases, ER-α, and ER-β and I learned how to design, run, and analyze gene expression using QPCR. I measured brain and gonadal aromatase, ER-α, and ER-β

in the brain, liver, and gonad of 15 hermaphrodites and 15 male mangrove rivulus and found some interesting results (see Figures 1 – 4).

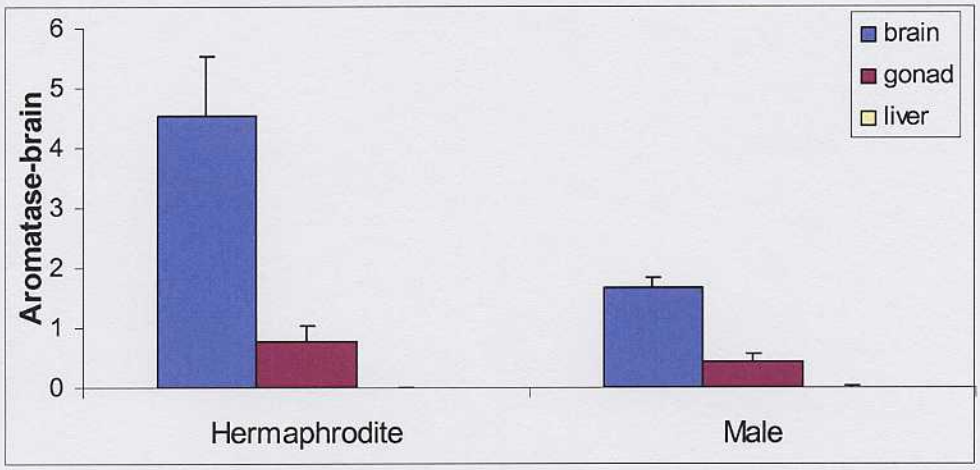

Figure 1. Brain aromatase gene expression (normalized with L8 ribosomal protein housekeeping gene) in hermaphrodite brains was more than 2.7 times greater than in male brains, while there were no differences between reproductive morphs in brain aromatase gene expression in the gonad or liver. Values shown are the mean ± 1 SEM and n = 15 ea.

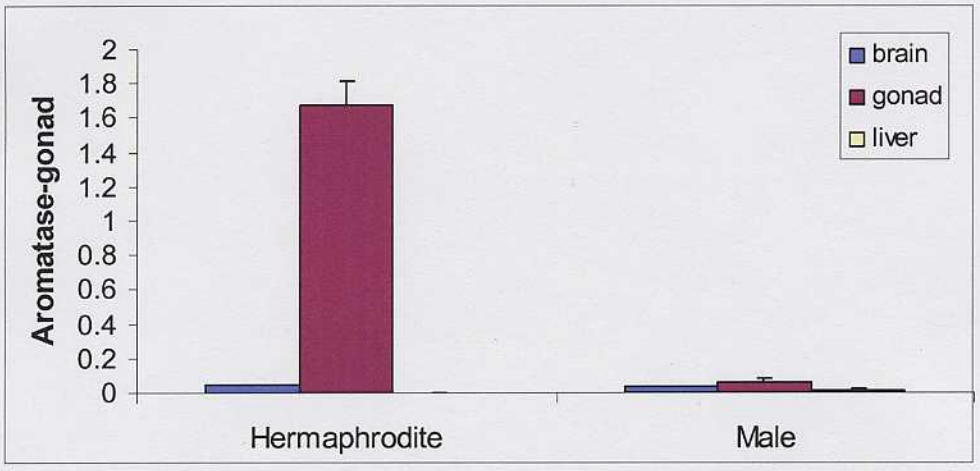

Figure 2. Gonadal aromatase gene expression (normalized with L8 ribosomal protein housekeeping gene) in hermaphrodite ovotestes was more than 17 times greater than in male testes, while there were no differences between reproductive morphs in gonadal aromatase gene expression in the brain or liver. Values shown are the mean ± 1 SEM and n = 15 ea.

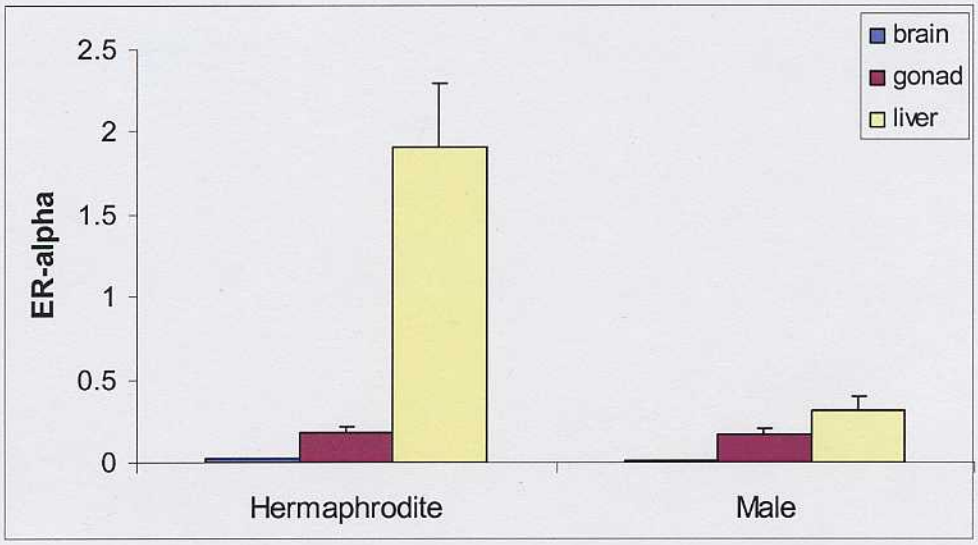

Figure 3. Estrogen receptor alpha gene expression (normalized with L8 ribosomal protein housekeeping gene) in hermaphrodite liver was more than 7.4 times greater than in male liver, while there were no differences between reproductive morphs in estrogen receptor alpha gene expression in the brain or gonad. Values shown are the mean ± 1 SEM and n = 15 ea.

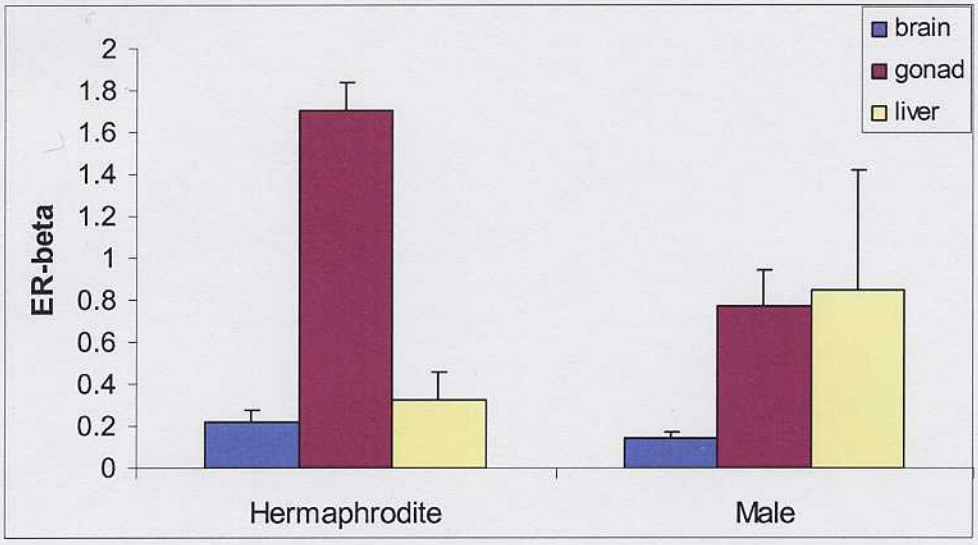

Figure 4. Estrogen receptor beta gene expression (normalized with L8 ribosomal protein housekeeping gene) in hermaphrodite ovotestes was more than 2.2 times greater than in male testes, while there were no differences between reproductive morphs in estrogen receptor beta gene expression in the brain or liver. Values shown are the mean ± 1 SEM and n = 15 ea.

The results of this initial study on gene expression in rivulus are both as *expected* (supported by the literature) and *intriguing* (interesting, but not because they are opposite to what one would reasonable expect). The increased (17X) gonadal aromatase gene expression in hermaphrodites vs. males makes sense due to the presence of ovarian tissue (Figure 2). The increased (7.4X) ER-α (Figure 3) and (2.2X) ER-β (Figure 4) gene expression in the livers and in the gonads, respectively, of hermaphrodites vs. males is expected, as hepatic vitellogenesis is known to be induced through the alpha isoform of the ER, whereas the beta isoform is more common in ovarian tissue.

The 2.7X increase in brain aromatase gene expression in the hermaphrodite vs. male brain is quite interesting and suggests a sexual dimorphism in the brain of a fish species that is not known to outcross, i.e., this species would seemingly not have a need for sexually dimorphic reproductive behavior (Figure 1). Given the rather high variation in the male data, there was no significant mean difference, but there was an interesting trend in the hepatic expression of the ER-β gene (Figure 4).

The research completed during this fellowship will contribute to the publication of two journal articles, one which is in preparation currently (Orlando, *et al.*, in preparation) and the second (dmrt-1 and fig-α) to follow in the near future. In a collaborative agreement with Drs. Katsu and Iguchi and Drs. Kanamori (Nagoya University, Japan) and Lee (Hanyang University, South Korea), we will examine dmrt-1 and fig-α expression in the hermaphrodites and male tissue that I left at Dr. Iguchi's lab. My hope is that the relationships begun during these few weeks will lead to rich collaborations in the future.

Outline of academic activities while in Japan (e.g., discussions, lectures, research)

My time in Japan was filled with research; however, I did enjoy conversations shared with other members of the Iguchi Lab both during the day and in the evenings during dinner (photo at right). Hajimi Watanbe, Ph.D. (associate professor) and I had enjoyed a couple of conversations over lunch and otherwise about DNA microarrays. Earlier this summer, I had taken a course at the Cold Spring Harbor Laboratory about microarrays and very much enjoyed our discussions about Dr. Watanabe's research on mouse reproductive tract gene expression. I shared with him my experience and some of the protocols we used in the course.

Kiyoaki Sone (research fellow) and I have common research interests in a fish species that not many people have worked on, and it was exciting to have the chance to meet and interact in person with him. We share an interest in how environmental chemicals affect the sexual differentiation of mosquitofish, *Gambusia affinis* and *Gambusia holbrooki* and discussed future possible collaborative research.

Also, I took a day to travel to Nagoya University to meet Akira Kanamori, Ph.D. (at right in the photo opposite, an assistant professor in the lab of Professor Hiroshi Hori, at left in the photo opposite). Akira and I share an interest in the mechanisms underlying sex determination and differentiation of fishes and he has worked on the mangrove rivulus as well. Coincidently, we have both done very similar experiments and there was a possibility of overlap in our studies. In Nagoya, we agreed to divide our current projects, and in the future, to collaborate and co-publish wherever practical. Since my time in Japan, we have already been in communication by email and have a collaborative research project examining genetic variation within and between populations of rivulus and have been exchanging protocols and discussion results of some of our studies that overlap. To my knowledge, there are only about a dozen researchers world-wide who are working on the mangrove rivulus. Further, only Akira and I share an interest in the sexual development of this fish, so meeting him in person was a wonderful opportunity made possible by this fellowship. The photo above shows Professor Hori and Akira at dinner the evening of my visit to their lab in Nagoya. I will always remember the generous hospitality extended to me that day and hope that one day I can host them in the USA.

Impressions and thoughts on the present state of the science in Japan in your field

It is of course difficult for me to have an accurate opinion of the present state of science in Japan. What I can speak to are the fields of research which I am part of that include the reproductive physiology of fishes and how environmental chemicals affect the reproduction of fishes. From my perspective, Japanese researchers are either leading (reproductive physiology) or are at the forefront (endocrine disrupting chemicals) of both of these fields.

Comments or suggestions to JSPS, especially concerning the Fellowship program

A *terrific* program that I hope continues into the future. As more connections are made between people of different nations, more barriers are broken down, and the rate of the progress of science will increase to the benefit of all scientists and the nations who support JSPS fellowships and similar programs.

Other comments

As I told Professor Iguchi, the only negative aspect of my fellowship was that now that I have been to the NIBB in Okazaki, I am spoiled for life! I am very impressed by the facility, and especially by the efficiency and hard-working nature of everyone at the Iguchi Lab. I respect hard work and getting results from the work one does. It is clear to me from the time I spent in his lab, and the talks by Professor Iguchi that I have attended, that his lab is one to emulate.

Although I will probably never have as large a lab as he, it was quite interesting for me to experience how a lab in Japan runs and I learned some management techniques that I will take home to my own lab. This was a bonus that I was not expecting from my fellowship.

Literature Cited

1. **Harrington, J., R W.** 1961. Oviparous hermaphroditic fish with internal self-fertilization. Science 134:1749-1750.
2. **Harrington, J., R W.** 1975. Sex Determination and Differentiation Among Uniparental Homozygotes of the Hermaphroditic Fish *Rivulus marmoratus* (Cyprinodontidae: Atheriniformes), pp. 249-262. *In* R. Reinboth (ed.), Intersexuality in the Animal Kingdom. Springer-Verlag, New York.
3. **Orlando, E. F., Y. Katsu, S. Miyagawa, and T. Iguchi.** In preparation. Differential expression of ER-α, ER-ß, aromatase-a, and aromatase-b in the brain, liver, and gonad of self-fertilizing hermaphroditic and male reproductive morphs of the mangrove rivulus, *Rivulus marmoratus*. *For:* Nature.
4. **Taylor, D. S.** 2000. Biology and ecology of *Rivulus marmoratus*: new insights and a review. Florida Scientist 63:242-255.
5. **Turner, B. J., W. P. Davis, and D. S. Taylor.** 1992. Abundant males in populations of a selfing hermaphroditic fish, *Rivulus marmoratus*, from some Belize cays. Journal of Fish Biology 40:307-310.